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14. ABSTRACT
Tumor suppressor gene methylation is an early step-wise change in benign breast epithelium undergoing neoplastic transformation. Preliminary studies suggest that quantification of DNA methylation in benign random perareolar fine needle aspiration (RP-FNA) samples may provide a marker of breast cancer risk. We assessed the technical performance characteristics of QM-MSP in RP-FNA using an 11-marker panel and performed a case control study that included 31 breast cancer patients and 130 community-based controls. Though DNA methylation does provide a modest risk signal, high inter-assay variability and poor reproducibility make it unattractive for clinical risk stratification in its current form.

15. SUBJECT TERMS
Benign breast epithelium, DNA methylation, Breast cancer risk

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1. **INTRODUCTION:** Tumor suppressor gene silencing by promoter region hypermethylation is an early step-wise event in breast carcinogenesis that is relevant to nearly all breast cancers[1]. Its occurrence in benign proliferative disease[2,3,4,5],[6] and in benign breast tissue near breast cancers [4,7]suggests that it may occur as a field change that could be exploited for individual breast cancer risk stratification. In 2005, using a qualitative methylation assay, we reported a great frequency of methylation in genes like RASF1A in random periareolar fine needle aspiration (RP-FNA) samples from women at increased risk for breast cancer compared to lower risk woman[8]. This observation was confirmed in an independent sample set using a quantitative methylation assay[9], but the risk signal afforded by our candidate gene list was too small for immediate clinical application. We hypothesized that a better gene list would provide better risk stratification and embarked on an unbiased whole genome search for markers that were more frequently and intensely methylated in benign breast tissue samples from women with breast cancer than unaffected women. We identified several new markers[10], and initial work in a training sample set suggested excellent discrimination between benign RP-FNA samples from breast cancer patients and unaffected women. This discrimination was not reproduced in an independent validation sample set because of higher methylation signals in the clinic-based control group than we had previously observed. This extension grant was requested to accrue an unselected, community-based control group, to evaluate additional candidate markers from our whole genome screen, and to evaluate the performance characteristics of methylation assays in RP-FNA samples that would influence future clinical utility for risk stratification.

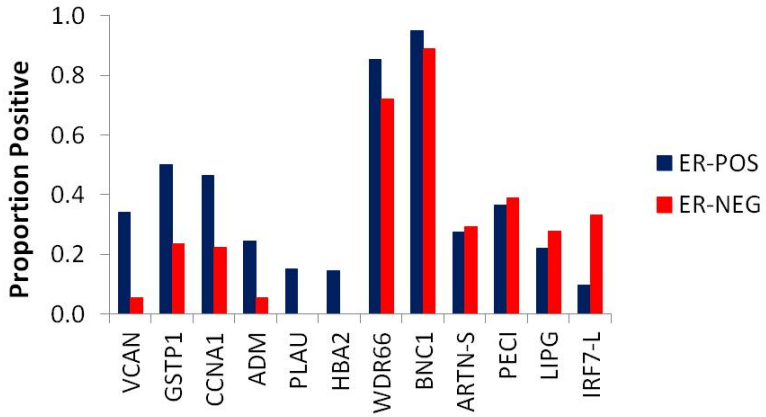
2. **KEYWORDS:** Breast Neoplasms, Benign Breast, DNA Methylation, Tumor Suppressor Genes, Fine Needle Aspiration Biopsy, Epigenetics.

3. **OVERALL PROJECT SUMMARY:**

Task 1: Assess the potential of new markers for epigenetic breast cancer risk stratification

Our initial DoD grant used unbiased whole genome and candidate gene approaches to identify 284 breast cancer methylation markers. We reduced this list to 63 genes by eliminating those that were methylated in lymphocytes (this would interfere with a clinical test based on random periareolar fine needle aspiration [RP-FNA] samples), and those that were generally methylated in all epithelial cells using a comparatively low sensitivity assay (MSP). We have previously thoroughly evaluated 17 of these genes and one aim of this extension was to thoroughly evaluate additional genes. We started by measuring methylation prevalence in breast cancers according to estrogen receptor status. As with most any gene set that has been previously evaluated, methylation is more frequent in ER(+) than ER(-

Figure 1: DNA Methylation in Breast Cancer by ER Status



) cancers. The only exception was IRF7 which was methylated more frequently in ER(-) than ER(+) cancer. Next we assessed these markers in a large archival RP-FNA sample set looking for markers that met the following criteria: 1) Methylated much more frequently in breast cancer than any benign samples, 2) methylated at greater frequency and intensity in benign breast cells from women with a newly diagnosed breast cancer than similar women never diagnosed with breast cancer, and 3) methylation in benign breast RP-FNA predicts methylation in an associated cancer. None of the genes met all of these criteria, PECI and GSTP1 fulfilled most so were advanced for further assessment. IRF7 and VCAN were also retained because of the strong association with ER(+) and ER(-) breast cancer respectively. The 2012 Annual report includes detailed data about the 12 new genes we evaluated for this task. At this point we systematically reviewed all of the data we had from the initial funding period and the extension period in order to construct a multi-gene panel with the greatest probability of being able to discriminate between benign RP-FNA samples from recently diagnosed breast cancer patient and similar samples from women never diagnosed with breast cancer. The final 11-gene panel included: PECI, IRF7, VCAN, GNE, PSAT1, HS3ST2, CCND2, WDR66, GSTP1, APC, and RASF1A.

Task 2: Establish epigenetic regulation of gene expression by promoter region hypermethylation for all of the genes in the final panel.

Ample published data establishing epigenetic regulation of gene expression by promoter region hypermethylation was available for all of the genes in our final 11-marker panel except PECI. PECI is likely to have escaped notice in prior studies, because it does not show high levels of methylation in most breast cancers. It is possible that minor cell populations are more important than bulk cell populations for predicting breast cancer risk or for predicting the behavior of established tumors. Analysis of archival samples (shown in Figure 2), suggested that PECI is not differentially methylated in breast cancer as compared to benign breast tissue, but it caught our attention because methylation in benign breast cells was highly predictive of methylation in the associated breast cancers and there was a hint of a signal for differentiating between benign RP-FNA samples from breast cancer patients and controls.

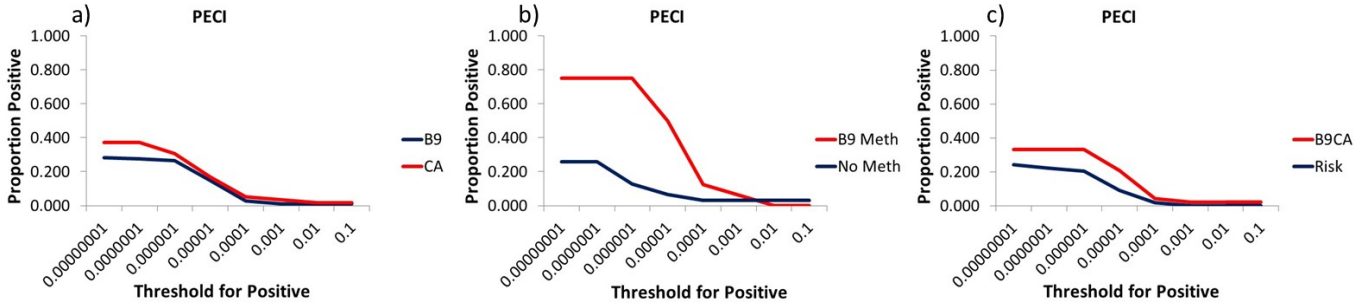


Figure 2: a) PECI is methylated in a small subpopulation of tumor cells in about 40% of breast cancers and 30% of benign samples. b) PECI methylation in a benign sample is highly predictive of PECI methylation in an associated breast cancer. c) PECI was more frequently methylated in benign RP-FNA samples from breast cancer patients than controls, though the difference was not large.

To determine whether PECI expression is epigenetically regulated by promoter region methylation, we selected 6 breast cancer cell lines – three that were methylated by QM-MSP and

three that were not methylated. These 6 cell lines were cloned and the the PECI promoter regions of the clones sequenced after sodium bisulfite treatment. Next, PECI mRNA expression was assessed in these cell lines using RT-PCR after treatment with DMSO or 1 μM 5-aza-2'-deoxycitidine. Finally PECI protein expression was assessed by Western blotting after treatment with DMSO or 5AZA. Results are shown in Figure 3.

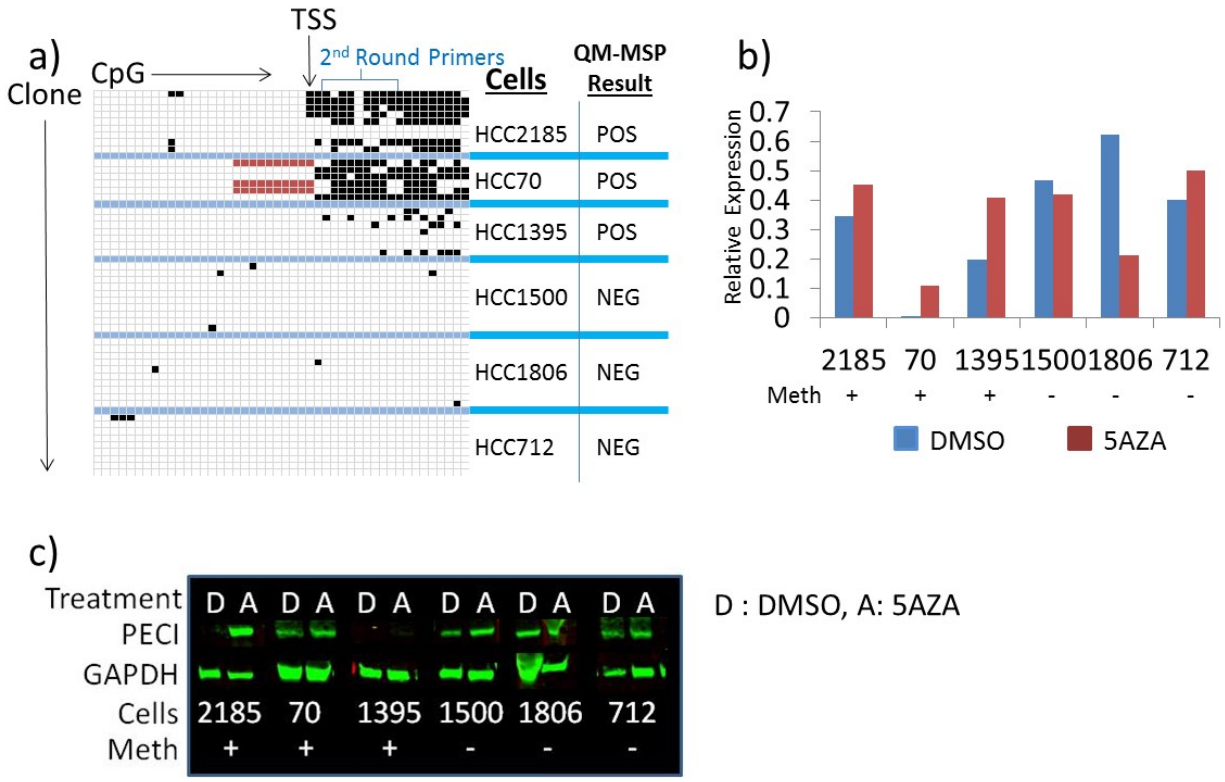


Figure 3: a) The cell lines we had identified as methylated by QM-MSP clearly show methylation of CpG's near the transcription start site, in the region assessed by the second round primers, while lines classified as negative for methylation did not. Methylation was quite dense for HCC2185 and HCC70. This figure confirms that the region we had selected for primer design is the correct region to assess. b) Baseline PECI mRNA expression is considerably lower for the methylated lines HCC70 and HCC1395 than for the unmethylated lines and 5AZA treatment was clearly associated with induction of PECI mRNA expression for these line. The baseline level of PECI mRNA expression for the methylated cell line HCC2185 was similar to the that of the unmethylated HCC712 line and showed only a marginal increase with 5AZA . c) Western blotting clearly shows a complete absence of PECI protein expression for HCC2185 at baseline with strong induction by 5AZA. HCC70, the other densely methylated cell line, shows low levels of PECI expression at baseline and after DSMO treatment . HCC1395, which had shown induction of PECI mRNA by 5AZA did not produce enough protein to be detectable by Western blot.

Interpretation: Promoter region hypermethylation is one mechanism by which breast cancer cells silence PECI expression (HCC2185), but PECI can be silenced by other mechanisms as well (HCC1395). Promoter region methylation of PECI is not always associated with loss of protein expression (HCC70).

Task 3: Determine the performance characteristics of DNA methylation analysis in RP-FNA samples.

With an eye towards eventual clinical application of DNA methylation analysis in RP-FNA samples we systematically explored issues related to sample processing, sample stability and reproducibility.

3a. Intra-assay and Inter-assay Reproducibility

We have previously reported intra-assay and inter-assay reproducibility for a panel of 5 genes assessed by quantitative multiplex MSP[11]. Intra-assay coefficients of variation ranged from 0.148 to 0.436 for samples with methylation of <1% of gene copies and from 0.003 to 0.305 for samples with methylation of >80% of gene copies depending on the specific gene assessed. Inter-assay coefficients of variation ranged from 0.159 to 0.555. For RASF1A, which had the lowest inter-assay reproducibility, a methylation fraction of 0.06 would fall 2 SDs above a methylation fraction of 0.03, permitting reliable discrimination between these values. However, assay reproducibility poses a challenge for the lower levels of methylation we are interested in for breast cancer risk stratification.

3b. Effects of Sample Storage

We compared quantitative methylation values measured for 3 genes in freshly processed benign RP-FNA samples with those measured up to 12 years later in sodium bisulfite-treated DNA stored at -20^{o}C. QM-MSP was used for both time points, but the second time-point assay was not identical to the initial assay because of changes in Taq and probe vendors and mixtures.

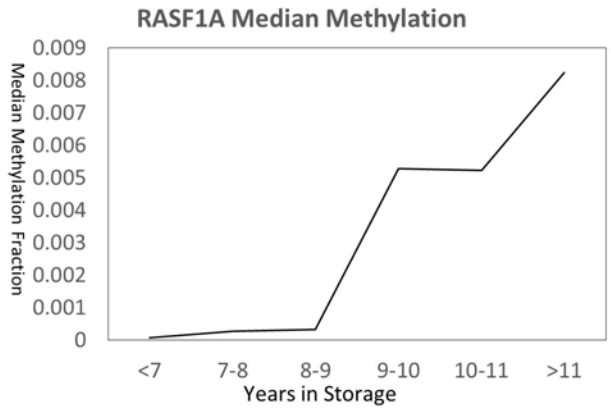
Table 1: Correlation between methylation assays run on the same samples in fresh or stored samples.

Gene	N Pairs	Spearman R	P-Value
APC	117	0.514	<0.0001
RASF1A	113	0.254	0.007
CCND2	112	0.305	0.001

There was statistically significant correlation between the two time points, however correlation is not the best measure when the majority of samples have very low levels of methylation. To account for this we calculated the probability that a second time point sample would be greater than median for all of the second time point assays if the first time point sample was greater than median for those assays.

Table 2: Odds that both the T1 and T2 sample will be > median for their respective time points

Gene	T1 median	T2 Median	OR (95% CI)	P-Value
APC	0.00001	0	17.98 (6.191 to 52.23)	<0.0001
RASF1A	0.00001	0.00316	3.499 (1.588 to 7.708)	0.002
CCND2	0.00001	0.002615	1.947 (0.9089 to 4.172)	0.085



APC showed considerable stability over time, but RASF1A and CCND2 did not. Next we focused on RASF1A to determine how methylation values were changing over time. RASF1A measured in stored sodium bisulfite-treated DNA was stable for about 9 years, but gradually drifted upward after that. This suggests that sequences with cytosines that had been converted to uracil by sodium bisulfite treatment may degrade over time.

Figure 4: Changes in median RASF1A methylation in sodium bisulfite-treated DNA from benign RP-FNA samples stored at -20ºC.

3c. Initial Sample Collection and Processing

For all of our DNA methylation studies we have prepared DNA from RP-FNA samples expelled directly into a very small volume (200 µl) of methanol-water solution (PreservCyt). These samples will contain a mixture of epithelial cells, stromal cells, adipose cells, cell-free DNA, and blood. We have accounted for potential monocyte contamination by carefully selecting markers that have no detectable methylation in lymphocytes. As part of this project we obtained RP-FNA samples from Carol Fabian. Dr. Fabian expels her RP-FNA samples into 9 ml of methanol-water solution (CytoLyt) with 1 ml of 10% neutral buffered formalin. After 24 hours, the cells spun, washed, and resuspended in PreservCyt which is used to make slides using the ThinPrep™ system (Cytyc Corporation) which filters the liquid to capture the cells. Dr. Fabian provided us with ThinPrep slides from 60 benign RP-FNA samples. We soaked the coverslips off with xylene and then scrapped the cells into Eppendorf tubes for DNA extraction and sodium bisulfite treatment using our standard protocols. We examined the prevalence of methylation of 11 genes for the ThinPrep samples as compared to our standard samples.

Table 3: Methylation prevalence for 11 genes by initial sample collection protocol.

Gene	% slides with result	[a]Slides	[b]PreservCyt (our standard protocol)
PECI	0.98	0.75	[c]0.24
IRF7	0.95	0.04	[c]0.09
VCAN	0.77	0	[c]0
GNE	0.8	0.06	[d]0.01 - 0.05
PSAT1	0.75	0	[d]0.14 – 0.51
HS3ST2	0.83	0.02	[d]0.14 - 0.38
CCND1	0.85	0.31	[d]0.33 - 0.66
WDR66	0.8	0.1	[c]0.89
GSTP1	0.77	0	[c]0.22
APC	0.85	0.16	[e]0.1 – 0.25
RASF1A	0.9	0.19	[e]0.1 – 0.35

[a]Proportion of samples with any detectible methylation
[b]Available prior datasets
[c]DoD 2010 ExtensionT raining set data
[d]DoD 2006 Training and Validation data
[e]CEBP 2008 Gail low risk – Gail high risk[9]

ThinPrep slides provided good quality DNA suitable for the QM-MSP assay. Methylation prevalence was similar between the two sample sources for IRF7, VCAN, GNE, APC and RASF1A. We have consistently observed fairly high methylation prevalence in benign RP-FNA samples for WDR66, PSAT1, and HS3ST2, but methylation prevalence for these genes was quite low in the ThinPrep slides. This suggests that the methylation we are detecting may be from some source other than intact epithelial cells. Also surprising was the high frequency of PECI methylation in the ThinPrep samples. This suggests that the ThinPrep process may enrich for the PECI-methylated cell population. The conclusion is that initial sample processing does significantly impact the final results.

3d. Reproducibility of 2 Time Point Samples

Next we looked at the stability of methylation results in individuals who had undergone RP-FNA sampling at two time points. We identified 40 subjects who had undergone RP-FNA sampling at two time points and Dr. Fabian provided 30 cases giving 70 sample pairs. We initially analyzed these sample sets separately, but the results were very similar so we are only showing the combined analysis below. The median interval between the two samplings was 0.77 years (9.2 months) with a range of 0.22 – 1.73 years. The median age of the first time point sample (i.e. time in storage) was 7.7 years with a range of 6.3 – 12.9 years.

QM-MSP for the full 11-marker panel was run on each of the samples. We have always observed that some patients show methylation of multiple genes while others show no methylation at all. We first asked whether subjects with higher average methylation for the 11 markers at the first time point also had higher average methylation at the second time point. Next we looked at individual genes. To do this, we selected threshold values and asked, if the first time point sample exceeded this threshold what is the probability that the second time point also exceeded it as compared to the probability that the second time point sample exceeded the threshold when the first sample did not.

Table 4: Stability of Methylation Results for Samples Obtained at Different Times in the Same Subjects

Threshold	[a]%T1	[b]If T1(+)	[c]ifT1(-)	P-value
Average for all 11 markers				
>0.001	0.9	0.952	0.25	0.0016
>0.01	0.729	0.686	0.389	0.047
RASF1A Combined Liquid and Slide				
>0	0.556	0.848	0.259	<0.0001
>0.001	0.19	0.5	0.128	0.01
>0.01	0.127	0.375	0.132	0.115

[a]Proportion of first time point samples exceeding the threshold
[b]Proportion of second time point samples exceeding the threshold when the first time point sample exceeded it.

[c]Proportion of second time point samples exceeding the threshold when the first sample did not

In general, the methylator phenotype was preserved between the two time points. Of the 11 markers assessed, only RASF1A showed consistent statistically significant reproducibility between the two time points.

3e. Effects of Tamoxifen on DNA Methylation in Benign RP-FNA Samples

We had previously conducted a randomized prospective placebo-controlled clinical trial to identify biomarkers in benign breast tissue that are modulated by tamoxifen but not placebo[12]. Subjects underwent RP-FNA sampling at baseline and after 3 months of treatment with tamoxifen or placebo. We had observed a modest decrease in methylation of RASF1A and APC for the tamoxifen-treated subjects, but not the controls. These RP-FNA samples were reassessed using our 11-marker panel.

Table 5: Changes in DNA Methylation Associated with Tamoxifen Treatment.

Gene	N Pairs	Mean Baseline	Mean Treat	P-Value
PECI	36	0.09375	0.06702	0.219
IRF7	36	0.04619	0.00003197	0.25
VCAN	33	0.02775	0.03378	0.508
GNE	34	0.002799	0.002384	0.861
PSAT1	32	0.03213	0.04027	0.536
HS3ST2	27	0.03253	0.03078	0.9
CCND2	32	0.04991	0.01618	0.224
WDR66	33	0.1794	0.1673	0.322
GSTP1	29	0.02391	0.004196	0.039
APC	35	0.08575	0.08096	0.812
RASF1A	32	0.07742	0.05014	0.115

[a] Wilcoxin Matched Pairs Sign Rank Test

Tamoxifen treatment was associated with reduced methylation of PECI, IRF7, CCND2, GSTP1, and RASF1A, but these results were only statistically significant for GSTP1. We think it is likely that tamoxifen does reduce certain methylated cell populations, but the effect is modest at 3 months.

3f. Benign RP-FNA Methylation in Women who Subsequently Developed Breast Cancer

We identified 10 women who developed breast cancer a median of 2.4 years after benign RP-FNA sampling (range = 1.0 – 5.5 years). We selected two age-matched controls for each of these women and asked whether those who developed breast cancer had higher methylation levels than the controls.

Table 6: DNA Methylation in Women who Subsequently Developed Breast Cancer

Gene	Mean CA	Mean Control	P-value
PECI	0.082	0.047	0.484
IRF7	0.082	0	0.174

VCAN	0.016	0	0.149
GNE	0	0.03	0.516
PSAT1	0.0194	0.016	0.878
HS3ST2	0.038	0.0007	0.085
CCND2	0.006	0.021	0.478
WDR66	0.148	0.074	0.027
GSTP1	0.043	0.022	0.335
APC	0.107	0.232	0.191
RASF1A	0.078	0.077	0.98
[a]MethSum	0.603	0.492	0.512
[b]MeanMeth	0.055	0.045	0.486
%Mark>0.01	0.349	0.268	0.26
[a]Sum of all the markers [b]Average of all the markers			

The number of observations is quite small and no firm conclusion can be drawn. Surprisingly, WDR66 was highly statistically significant.

Task 4: Initiate a prospective epigenetic testing registry for unaffected women.

Our primary goal has been to identify markers that are more frequently and intensely methylated in benign RP-FNA samples from women recently diagnosed with breast cancer than women never diagnosed with breast cancer. Our prior case-control studies were hampered by lack of a truly unselected control group. Historically, our control groups were drawn from the clinic and often had family histories of breast cancer or personal histories of high risk preneoplasia. The intent of this task was to accumulate an unselected community-based control group. This was successful based on the 130 RP-FNA samples we were able to collect from community volunteers. Table 7 shows the characteristics of these community-based control subjects as compared to 31 newly diagnosed breast cancer patients sampled during the same time period. Only one subject had a prior history of high risk preneoplasia.

Table 7:			
	Cancer	Control	P-value
Number	31	130	
Mean Age (range)	58 (40-77)	61 (33-79)	0.148
Mean BMI (range)	39.3 (16.3-48.5)	33.1 (18.4-53.4)	0.01
Race/Ethnicity (%)			0.157
White	21 (68)	108 (83)	
Black	6 (19)	13 (10)	
Hispanic	0	7 (5)	
Asian	3 (10)	2 (2)	
Unknown	1 (3)	0	
Premenopausal (%)	8 (26)	21 (16)	0.209
Nulliparous (%)	2 (6)	33 (25)	0.027
Median Parity (for parous)	2	2	0.80
Mean Age First Live Birth	24.6	23.9	0.568
Prior B9 Biopsy (%)	9 (29)	43 (33)	0.665

FHX FDR Breast Cancer (%)	6 (19)	36 (28)	0.388
Any FHX Breast Cancer	13 (42)	64 (49)	0.281
Mean 5 Yr Gail Risk	1.63	2.05	0.086
FHX FDR: Family history of breast cancer in a first degree relative			

There is some evidence of self-selection among the community volunteers. They were mostly white, postmenopausal women and enriched for nulliparous women and women with a family history of breast cancer in a first degree relative. The mean 5-year Gail risk of 2.05% is not far above what would be expected as the 5-year Gail risk for the average 60 year old Caucasian woman is 1.7%.

The 11-marker panel was assessed in all of the benign RP-FNA samples from the cancer patients and the unaffected community-based controls. The panel was also run on FNA samples from the tumors of the cancer patients. Figure 5 shows the distribution of methylation values across a wide range of detection thresholds (10^{-8} – 0.01). The left side of each curve would correspond to detection of minor cell populations and the right to bulk populations.

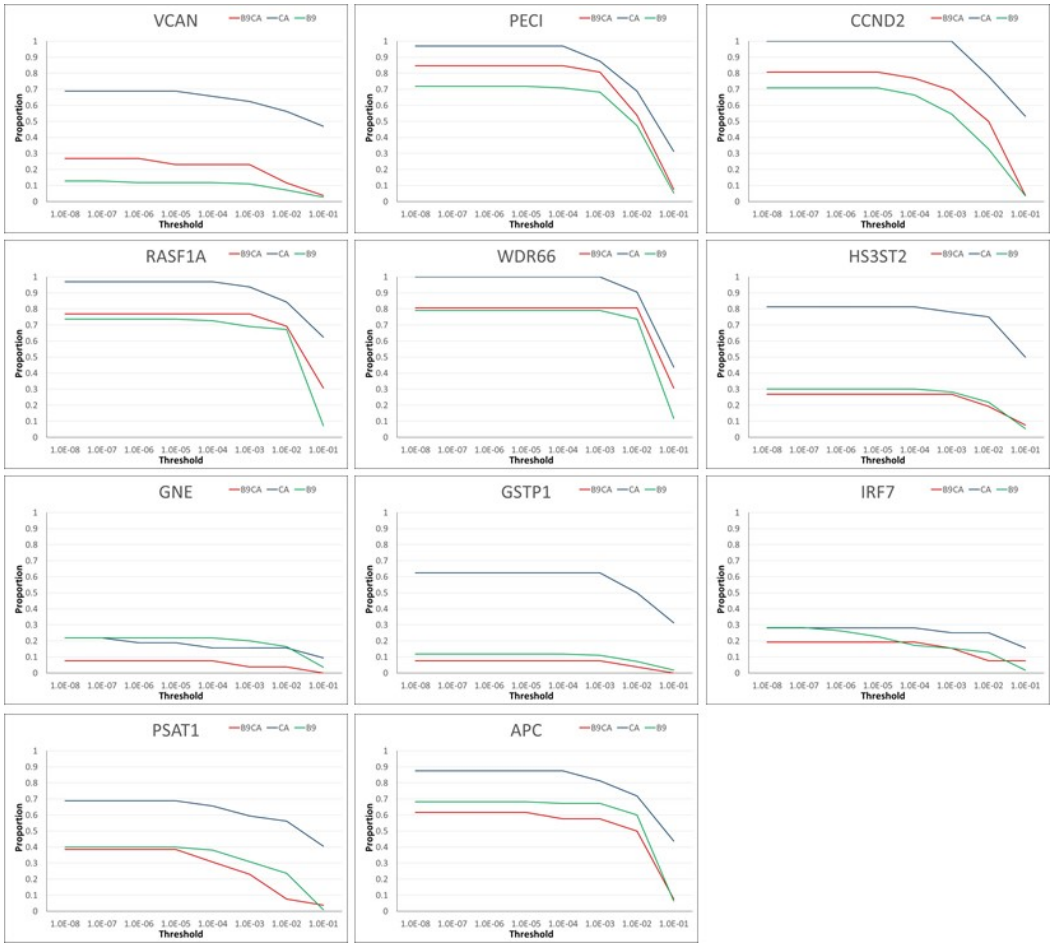


Figure 5: Quantitative methylation results for 11-genes assessed in primary breast cancer FNA's (blue lines), benign RP-FNA's from breast cancer patients (red lines), and benign RP-FNA's from an unaffected community-based control group.

We had previously observed higher levels of methylation in benign RP-FNA samples from breast cancer patients than similar samples from unaffected women for CCND2, RASF1A, APC, HS3ST2 and PSAT1. Those observations are modestly reproduced for CCND2 and RASF1A (higher methylation threshold), but not the other genes. Of the new markers, VCAN, PECI, and WDR66 (higher methylation threshold) exhibit a signal in the desired direction, but the signal is not strong enough for a clinically viable test.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Identified IRF7 as gene that is more frequently methylated in ER(-) than ER(+) breast cancer.
- Established that PECI expression can be regulated by promoter region hypermethylation but other mechanism are likely operative as well in some cell lines.
- Inter-assay variation is high for QM-MSP assays, is gene specific, and is influenced by storage times. Methylation values gradually rise when sodium bisulfite-treated DNA is stored for > 8-9 years.
- The pattern of DNA methylation in benign breast cell samples can be very different depending on how the RP-FNA samples were initially processed. The ThinPrep method may provide the most relevant starting material.
- Multiple RP-FNA samplings in the same individual will not yield comparable methylation results for most genes. RASF1A may be an exception, and there is some evidence that women with generally higher levels of methylation across multiple genes on one sample will show the same on a second sample.
- We could not confirm a previous observation suggesting that 3 months of tamoxifen reduces APC and RASF1A methylation. Three months of tamoxifen does not exert any large effects on RP-FNA methylation.
- WDR66 methylation was significantly higher in benign RP-FNA samples from women who later developed breast cancer than age-matched control women who di not develop breast cancer but the number of observations is too small to draw firm conclusions.
- It is possible to accrue to a community-based RP-FNA repository, but the volunteers will have self-selected for certain risk factors such as family history of breast cancer in a first degree relative or nulliparity.
- The risk signal generated by DNA methylation in benign RP-FNA samples is too weak to provide a clinical useful breast cancer risk stratification test.

5. CONCLUSION:

There are persistent, modest signals suggesting that tumor suppressor gene methylation identified in benign breast RP-FNA samples has some relationship to increased breast cancer risk. High inter-assay variability and poor reproducibility for two time point samples overwhelms the risk signal making it unlikely that it will be tenable for clinical breast cancer risk stratification. There are still no validated breast tissue markers of increased breast cancer risk that can serve as surrogate endpoint biomarkers for breast cancer prevention studies. The best chance of finding markers will come through continued investment in prospective breast tissue

repositories, but only after these repositories have matured enough to provide sufficient numbers of post-sampling breast cancers.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:
Nothing to report

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report

8. REPORTABLE OUTCOMES: Nothing to report

9. OTHER ACHIEVEMENTS: Community-based breast RP-FNA repository.

10. REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

1. Novak P, Jensen TJ, Garbe JC, Stampfer MR, Futscher BW. Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. *Cancer Res.* Jun 15 2009;69(12):5251-5258.
2. Parrella P, Poeta ML, Gallo AP, et al. Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. *Clin Cancer Res.* Aug 15 2004;10(16):5349-5354.
3. Lehmann U, Langer F, Feist H, Glockner S, Hasemeier B, Kreipe H. Quantitative assessment of promoter hypermethylation during breast cancer development. *American Journal of Pathology.* Feb 2002;160(2):605-612.
4. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. *Oncogene.* 2001;20:3348–3353.
5. Jeronimo C, Costa I, Martins MC, et al. Detection of gene promoter hypermethylation in fine needle washings from breast lesions. *Clin Cancer Res.* Aug 15 2003;9(9):3413-3417.
6. Fackler MJ, McVeigh M, Evron E, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int J Cancer.* Dec 20 2003;107(6):970-975.
7. Li Z, Meng ZH, Chandrasekaran R, et al. Biallelic inactivation of the thyroid hormone receptor beta1 gene in early stage breast cancer. *Cancer Res.* 2002;62:1939–1943.
8. Lewis CM, Cler LR, Bu DW, et al. Promoter hypermethylation in benign breast epithelium in relation to predicted breast cancer risk. *Clin Cancer Res.* Jan 1 2005;11(1):166-172.
9. Euhus DM, Bu D, Milchgrub S, et al. DNA methylation in benign breast epithelium in relation to age and breast cancer risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* May 2008;17(5):1051-1059.
10. Bu D, Lewis CM, Sarode V, et al. Identification of breast cancer DNA methylation markers optimized for fine-needle aspiration samples. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* Dec 2013;22(12):2212-2221.

11. Euhus DM, Bu D-w, Ashfaq R, et al. Atypia and DNA Methylation in Nipple Duct Lavage in Relation to Predicted Breast Cancer Risk . *Cancer Epi Biomark Prev.* 2007;16:1812-1821.
12. Euhus D, Bu D, Xie XJ, et al. Tamoxifen downregulates ets oncogene family members ETV4 and ETV5 in benign breast tissue: implications for durable risk reduction. *Cancer Prev Res (Phila).* Nov 2011;4(11):1852-1862.

11. APPENDICES: None